Cell, Vol. 72, 197–209, January 29, 1993, Copyright © 1993 by Cell Press

The Zinc Finger Transcription Factor Egr-1 Is Essential for and Restricts Differentiation along the Macrophage Lineage

Hung Q. Nguyen, Barbara Hoffman-Li b rmann, and Dan A. Li b rmann
Department of Biochemistry and Biophysics
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania 19104

Summary

We have isolated cDNA clones of myeloid differentiation primary response (MyD) genes, activated in the absence of de novo protein synthesis following induction for differentiation along either the macrophage or granulocyte lineage in human myeloblastic leukemia HL-60 cells. One cDNA clone of a primary response gene, expressed upon macrophage differentiation, encoded for Egr-1, a zinc finger transcription factor. The *Egr-1* gene was observed to be transcriptionally silent in HL-60 cells, but active in U-937 and M1 cells, the latter two being predetermined for macrophage differentiation. Egr-1 antisense oligomers in the culture media blocked macrophage differentiation in both myeloid leukemia cell lines and normal myeloblasts. HL-60 cells constitutively expressing an *Egr-1* transgene (HL-60Egr-1) could be induced for macrophage, but not granulocyte, differentiation. These observations indicate that expression of *Egr-1* is essential for and restricts differentiation of myeloblasts along the macrophage lineage.

Introduction

Terminal differentiation of animal cells involves the highly controlled progression of cells through successive stages of differentiation and growth arrest. Hematopoiesis is a profound example of this process, which perseveres throughout life, in which a hierarchy of progenitor cells in the bone marrow proliferates and differentiates along multiple as well as distinct cell lineages (for overview, see Wintrobe et al., 1981). Both the ability to induce differentiation of normal myeloid cells in culture (Bradley and Metcalf, 1966; Pluznik and Sachs, 1965) and the availability of differentiation-inducible myeloid leukemia cell lines (Sachs, 1987; Collins, 1987; Koeffler, 1983) make it possible to dissect and analyze myelopoiesis. This includes elucidating the players involved at the molecular level in normal myeloid differentiation and identifying lesions that can lead to leukemogenicity and its progression (Liebermann et al., 1980, 1981; Liebermann and Hoffman-Liebermann, 1989).

U-937 is a human monoblastic leukemia cell line that can be differentiated into macrophage-like cells by phorbol-12-myristate-13-acetate (PMA) (Sundstrom and Nilsson, 1976; Larsson et al., 1988). Unlike the lineage predisposed U-937, the human myeloblastic leukemia HL-60 cell line (Dalton et al., 1988; Gallagh r et al., 1979) can differentiate along both the monocytic and granulocytic lineages (for review see Collins, 1987). While PMA induces macrophage differentiation (Rovera et al., 1979), dimethyl sulfoxide (DMSO) stimulates granulocytic differentiation (Collins et al., 1978). Simultaneous treatment with both inducers results in macrophage differentiation (Liebermann et al., 1981). Furthermore, when DMSO is removed from HL-60 DMSO-derived granulocyte-like cells after 5 days and PMA is added, the cells switch from granulocytic to monocytic differentiation (Liebermann et al., 1981). M1 is a murine myeloblastic leukemic cell line capable of differentiating into macrophages when stimulated by various physiological inducers such as interleukin-6 (IL-6), leukemia inhibitory factor (LIF), or lung conditioned medium (LCM, containing both IL-6 and LIF; Hoffman-Liebermann and Liebermann, 1991; Lord et al., 1990b, 1991). In normal myeloblast-enriched bone marrow cells, LCM induces differentiation along both the macrophage and granulocyte lineages (Liebermann and Hoffman-Liebermann, 1989), whereas the myelopoietic factors, macrophage colony-stimulating factor (M-CSF) and granulocyte colony-stimulating factor (G-CSF), induce either macrophage or granulocyte differentiation, respectively (Metcalf, 1985; Clark and Kamen, 1987).

To identify the genes that may play a role in the regulation of hematopoietic cell differentiation, we have isolated cDNA clones of myeloid differentiation primary response (MyD) genes, activated in the absence of de novo protein synthesis, in HL-60 and M1 cells following induction for macrophage or granulocyte differentiation (Lord et al., 1990a, 1990b; this study). In the course of this work, the zinc finger transcription factor Egr-1 (Krox24, NGIF-A, Zif/268, Tis8) has been identified as a myeloid differentiation primary response gene, specifically induced upon HL-60 macrophage differentiation. *Egr-1* has been previously identified as a growth response gene in cultured cells and in response to B cell maturation (Sukhatme et al., 1987; Lau and Nathans, 1987; Christy et al., 1988; Lemaire et al., 1988; Varnum et al., 1989a; Seyfert et al., 1990). However, it is also induced during differentiation of nerve, bone, and myeloid cells (Milbrandt, 1987; Suva et al., 1991; Bernstein et al., 1991). The Egr-1 protein has been localized to the nucleus and shown to bind specifically to the consensus sequence 5′-GCGGGGGCG-3′ as well as to transactivate a promoter containing this sequence (Christy and Nathans, 1989; Lemaire et al., 1990; Cao et al., 1990). Recently, the human *Egr-1* gene was localized to human chromosome 5, in a region often deleted in patients suffering from therapy-induced acute myeloid leukemia (Sukhatme et al., 1988; Nagarajan et al., 1990).

In this work we have used both differentiation-inducible myeloid leukemia cell lines and normal myeloblasts, in conjunction with antisense oligodeoxynucleotides in the culture medium and stable transfection methodologies to investigate the role of *Egr-1* in myeloid cell development. It is shown that *Egr-1* is an immediate early differentiation response gene essential for macrophage development. In addition, using HL-60 cells genetically manipulated

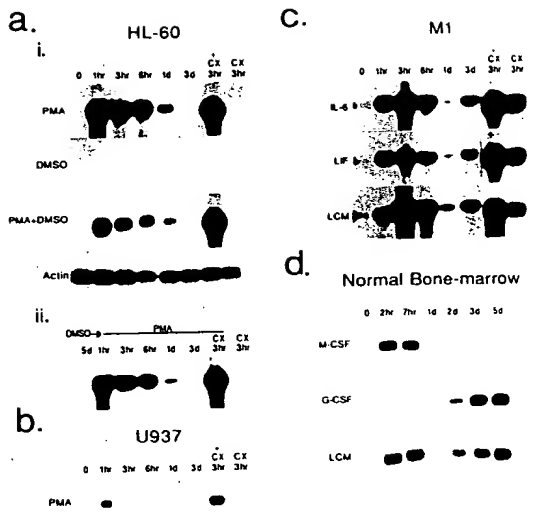


Figure 1. *Egr-1* Expression during Differentiation of Myeloid Leukemia Cell Lines and Bone Marrow–Derived Normal Myeloid Precursors

(a) *Egr-1* expression upon differentiation of HL-60 cells. HL-60 cells were treated with PMA (2 nM), DMSO (1.3%), or PMA (2 nM) plus DMSO (1.3%) for the indicated times (i), or for 5 days with DMSO (1.3%), after which time DMSO was removed and PMA (2 nM) was added for the indicated times (ii).

(b) *Egr-1* expression upon differentiation of U-937 cells. U-937 cells were induced for differentiation with PMA (100 nM).

(c) *Egr-1* expression upon induction of M1 differentiation. Murine M1 cells were treated with IL-6 (100 ng/ml), LIF (200 U/ml), or LCM (10 U/ml), as indicated in Experimental Procedures.

(d) *Egr-1* expression upon differentiation of myeloblast-enriched bone marrow cells. Myeloblast-enriched murine bone marrow cells were obtained from CD-1 mice and cultured as indicated in Experimental Procedures. Cells were stimulated with M-CSF (100 U/ml), G-CSF (160 ng/ml), or LCM (10 U/ml). Cells were seeded in the presence of inducers with (+CX) or without 10 μg/ml cycloheximide, or with cycloheximide only (CX); RNA blots were prepared with total RNA (10 μg per lane) and hybridized with ^{32}P-labeled probes specific for either the human (for HL-60 and U-937) or murine (for M1 and bone marrow) *Egr-1* gene. Resulting autoradiograms are shown. All the autoradiograms gave similar hybridization signals, comparable with what was shown in (a) (i), following hybridization to an actin probe.

to express an *Egr-1* transgene constitutively, it is shown that *Egr-1* restricts differentiation along the macrophage lineage.

Results

Egr-1, a Differentiation Primary Response Gene for Macrophage Development

A cDNA library was constructed using poly(A)$^+$ RNA from HL-60 cells stimulated with PMA for 3 hr in the presence of cycloheximide. Complex cDNA probes, synthesized from poly(A)$^+$ RNA of undifferentiated HL-60 cells and HL-60 cells stimulated with PMA for 3 hr in the presence of cycloheximide, were used to screen the cDNA library differentially to id ntify primary macrophage differentiation respons genes. A total of 100,000 clones were screened, and 230 were diff rentially expressed; 20 were plaque purified and partially sequenced. Homology searches against the GenBank data base with end sequences of the cDNA clone HLM-38 identified it as a cDNA for the zinc finger transcription factor Egr-1 (Sukhatme et al., 1988; Lemaire t al., 1988).

Expression analysis of *Egr-1* in HL-60 cells revealed that it is transiently induced during PMA-stimulated macrophage differentiation, with peak levels of expression at 1 hr (Figure 1a [i]). While it was superinduced with PMA in the presence of cycloheximide, it was not induced by cycloheximide alone. Similar kinetics of expression were seen in HL-60 cells stimulated simultaneously with PMA and DMSO (Figure 1a [i]). These inducers together gave rise to macrophage-like cells with a more dendritic morphology than PMA alone (Liebermann et al., 1981). HL-60 cells can be induced to become granulocyte-like cells in the presence of DMSO for 5 days, as assessed by morphology and nitroblue tetrazolium (NBT) staining (Collins et al., 1978; Torella et al., 1982). Interestingly, after 5 days, removal of DMSO and addition of PMA resulted in macrophage differentiation (Liebermann et al., 1981). As shown in Figure 1a (i), *Egr-1* was not expressed in DMSO-induced granulocyte-like cells after 5 days. Regardless of whether the original cells were HL-60 or HL-60-derived granulocyte-like cells, *Egr-1* was induced with indistinguishable kinetics when stimulated with PMA to undergo macrophage differentiation (Figure 1a [ii]). This included superinduction with PMA in the presence of cycloheximide and no induction by cycloheximide only (Figure 1a [ii]). In conclusion, *Egr-1* was expressed neither in uninduced HL-60 cells nor throughout granulocytic differentiation, but was expressed during macrophage differentiation.

Unlike HL-60 cells, U-937 cells are predisposed to differentiate along the monocytic lineage only. As shown in Figure 1b, uninduced U-937 cells did not express *Egr-1* mRNA, but it was induced with very transient kinetics when the cells were stimulated with PMA to undergo macrophage differentiation. Expression was high at 1 hr, followed by a decline to barely detectable levels at 3 hr. *Egr-1* mRNA was superinduced with PMA plus cycloheximide and was not detected following cycloheximide treatment for 3 hr (Figure 1b), but was detected following cycloheximide treatment for less than 1 hr (data not shown; Bernstein et al., 1991).

In contrast with HL-60 and U-937 cells, uninduced murine myeloblastic leukemia M1 cells expressed *Egr-1* mRNA, although at very low levels. As shown in Figure 1c, in M1 cells *Egr-1* expression exhibited biphasic kinetics of induction following treatment with the differentiation inducers IL-6, LIF, or LCM. The steady-state level of *Egr-1* mRNA peaked at 3 hr and declined to the uninduced level by 1 day, then increased about 10-fold by 3 days, when terminal macrophage differentiation occurs. As was the case for HL-60 and U-937, in M1 cells *Egr-1* expression was superinduced in the presence of both cycloheximide and differentiation inducers, and, as with U-937, cycloheximide by itself stimulated *Egr-1* expression (Figure 1c). *Egr-1* was not induced following treatment of M1 cells with IL-3, G-CSF, M-CSF, or GM-CSF, cytokines that neither induced M1 differentiation nor inhibited M1 cell growth.

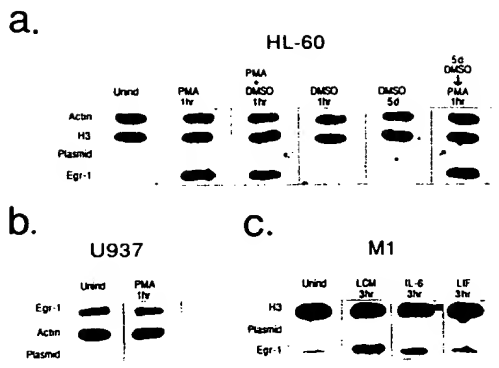


Figure 2. *Egr-1* Gene Transcription in HL-60, U-937, and M1 Cells

HL-60 cells shown in (a), U-937 in (b), and M1 in (c). Nuclear run-on transcription assays were performed as described in Experimental Procedures, with nuclei isolated from 5 × 10^7 cells before and after stimulation with differentiation inducers. HL-60 cells were treated for 1 hr with 10 nM PMA, 10 nM PMA plus 1.3% DMSO, or 1.3% DMSO. HL-60 cells were also stimulated with 1.3% DMSO for 5 days, after which time DMSO was removed and 10 nM PMA was added for 1 hr before isolation of nuclei. U-937 cells were induced with 100 nM PMA for 1 hr. M1 cells were stimulated with IL-6 (100 ng/ml), LIF (200 U/ml), or LCM (10 U/ml) for 3 hr. Radiolabeled nuclear run-on products were hybridized to nylon filters containing plasmid with or without inserts specific for *Egr-1* (either full length [murine] or corresponding to 2749–3060 bp [human]), β-actin, and/or H3 histone.

Egr-1 also was not induced following treatment of M1 cells with transforming growth factor β, interferon α, interferon β, or IL-1, cytokines that inhibited M1 cell growth but did not induce terminal differentiation (data not shown). Increased levels of *Egr-1* expression appears, thus, to be tightly linked to the induction of M1 terminal macrophage differentiation, but not growth arrest, induced by physiological factors.

Egr-1 expression was also analyzed in normal murine myeloblast-enriched bone marrow cells induced for differentiation with physiological inducers. Whereas M-CSF stimulates macrophage differentiation, G-CSF induces granulocytic differentiation. As seen in Figure 1d, *Egr-1* mRNA was not detectable in uninduced myeloblast-enriched bone marrow cells. Following stimulation with M-CSF, *Egr-1* was induced within 2 hr and continued to be expressed at 7 hr, then decreased to barely detectable levels by day 1 and remained undetectable at later times. In contrast with M-CSF, G-CSF induced detectable steady-state *Egr-1* transcripts only after 2 days, with a 20-fold increase observed by 3 days (Figure 1d), which was maintained at least through the 5th day following treatment. LCM, which contains a variety of myelopoietic cytokines including IL-6 and LIF (Hoffman-Liebermann and Liebermann, 1991), was shown to stimulate differentiation of bone marrow cells into both macrophages and granulocytes (Liebermann and Hoffman-Liebermann, 1989). When myeloblast-enriched bone marrow cells were treated with LCM, *Egr-1* expression exhibited biphasic kinetics (Figure 1d), resembling the superimpos d kinetics of xpr ssion obtained with M-CSF and G-CSF. This is consistent with the fact that LCM induced the development of both macrophages and granulocytes.

Taken together, the results of *Egr-1* expression analysis indicated that *Egr-1* is expressed early during macrophage differentiation of HL-60, U-937, and M1 cells, as well as upon macrophage differentiation of normal myeloid precursor cells. It is not expressed during granulocytic differentiation of HL-60 cells, but is expressed at late times during granulocytic differentiation of normal myeloid precursors. Sustained expression of *Egr-1* in mature cells was detected only with treatment by physiological inducers.

Regulation of *Egr-1* Gene Expression in Myeloid Differentiation

In light of the apparent macrophage lineage predetermination of M1 and U-937, but not HL-60 cells, we thought it would be interesting to determine the mode of induction of *Egr-1* expression in these cell lines. Toward this end, nuclear run-on assays were performed. The *Egr-1* gene was transcriptionally inactive in uninduced HL-60 cells (Figure 2a). Treatment with PMA or PMA plus DMSO, which induces macrophage differentiation, induced *Egr-1* expression at the transcriptional level, whereas treatment with DMSO alone, which induces granulocytic differentiation, for either 1 hr or 5 days did not. Removal of DMSO from the HL-60-derived granulocyte-like cells followed by addition of PMA, which results in macrophage differentiation, also resulted in transcriptional activation of *Egr-1*. Actin and the histone 3 genes are transcribed in uninduced cells, and their transcription rates remained invariant during macrophage or granulocyte differentiation (Figure 2a). The results clearly indicated that the induction of *Egr-1* steady-state transcripts during macrophage differentiation of HL-60 was by a transcriptional mechanism.

Nuclear run-on assays in U-937 disclosed that the *Egr-1* gene was transcribed in uninduced cells, although there were no detectable *Egr-1* steady-state transcripts (Figure 1b), and the transcription rate remained constant during macrophage differentiation induced by PMA (Figure 2b). Since the human *Egr-1* probe used was from the 3′ end of the cDNA (2749–3060 bp), the run-on assays demonstrated that the increase in *Egr-1* expression during macrophage differentiation was via a posttranscriptional mechanism. Consistent with this conclusion is the fact that treatment with cycloheximide for up to 1 hr stimulated *Egr-1* expression (data not shown; Bernstein et al., 1991).

As shown in Figure 1c, low levels of *Egr-1* steady-state transcripts were observed in uninduced M1 cells, with a massive (~60-fold) increase following induction for differentiation. While the transcription rate of the *Egr-1* gene in LCM-treated cells was noticeably increased from the uninduced cells, the increase in the transcription rate in IL-6- and LIF-treated cells was only slight (Figure 2c). However, for all the inducers, the increase in the transcription rate could not account for the increase in the steady-state *Egr-1* transcript levels. Thus, as in U-937 cells, the run-on assays and the stimulation of EGR-1 expression with cycloheximide (Figure 1c) suggested that the increase in the steady-state level of *Egr-1* mRNA following induction

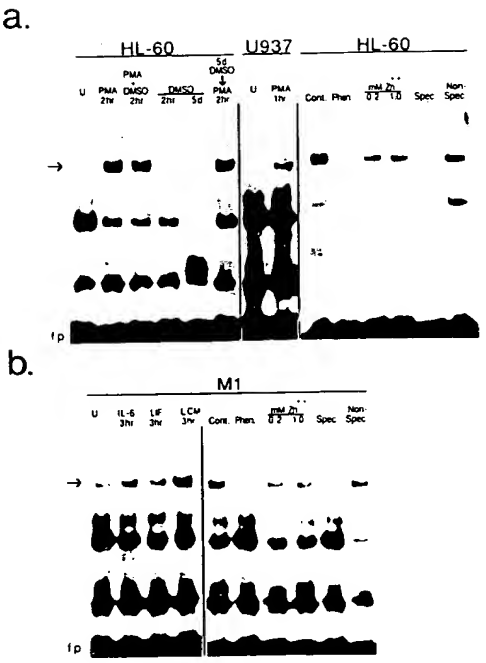


Figure 3. Gel Retardation Assays Using Total Nuclear Extracts from HL-60, U-937, and M1 Cells for *Egr-1* DNA Binding Activity

Extracts of nuclear proteins were prepared using 5 × 10^7 cells, before and after stimulation with differentiation inducers.

(a) *Egr-1* DNA binding activity in total nuclear extracts of HL-60 and U-937 cells. Extracts were prepared from HL-60 cells treated with 10 nM PMA, 10 nM PMA plus 1.3% DMSO, or 1.3% DMSO for 2 hr, and in the case of DMSO for 5 days, after which time DMSO was removed and 10 nM PMA was added for 2 hr. U-937 cells were induced with 100 nM PMA. For experiments to determine if the specific protein–DNA complex formation is dependent on zinc, HL-60 cells were induced to differentiate with 10 nM PMA and the nuclear extracts were untreated (Cont.) or pretreated with 1 nM 1,10-phenanthroline (Phen.), without or with the addition of 0.2 mM and 1 mM $ZnCl_2$ before the binding reaction. Competition experiments were accomplished using a 50-fold excess of unlabeled double-stranded specific (Spec.; Egr-1-binding sequence) or unrelated nonspecific (Non-Spec.; 5′-TCGACCGGATTA-CGTCATAGCTTCGA-3′) competitors in the binding reaction.

(b) *Egr-1* DNA binding activity in total nuclear extracts of M1 cells. M1 cells were treated with IL-6 (100 ng/ml), LIF (200 U/ml), or LCM (10 U/ml) for 3 hr before isolation of nuclei. Determining the dependence on zinc and specificity of binding sequences by competition experiments was as in (a) and the experiments are shown for extracts of M1 cells induced with LCM.

Arrows indicate the positions of the bands of interest, indicative of a specific protein–DNA complex and fp indicates the free probe.

of differentiation can be attributed mainly to regulation at a posttranscriptional level.

These data demonstrate that the *Egr-1* gene is transcriptionally silent in HL-60 cells, but active in U-937 and M1 cells, the latter two being predetermined for macrophage differentiation.

DNA Binding Function of the Zinc Finger Transcription Factor Encoded by the *Egr-1* Gene

It has been noted previously that HL-60 cells have a 5q deletion resulting in the absence of one of the allel s of the *Egr-1* gene (Nagarajan et al., 1990). Therefore, the question arises concerning the integrity of the other allele of the *Egr-1* gene. One criterion of functionality of the *Egr-1* gene product is its DNA binding capabilities. To examine the DNA binding of the zinc finger transcription factor encoded by the *Egr-1* gene, we employed gel retardation assays, using total nuclear extracts and ^{32}P-labeled oligodeoxynucleotide probes containing the Egr-1-binding site.

As shown in Figure 3a, a specific protein–DNA complex, indicated with an arrow, that was present in nuclear extracts from HL-60 cells induced for macrophage differentiation with PMA, PMA plus DMSO, or DMSO followed by PMA, was missing, in extracts from uninduced HL-60 cells and DMSO-treated HL-60 cells (either 2 hr or 5 days). The presence of the complex correlated with the presence of *Egr-1* transcripts. A protein–DNA complex not present in extracts from untreated U-937 cells is also present in extracts from PMA-treated U-937 cells. The migration of the U-937 complex was indistinguishable from the HL-60 complex.

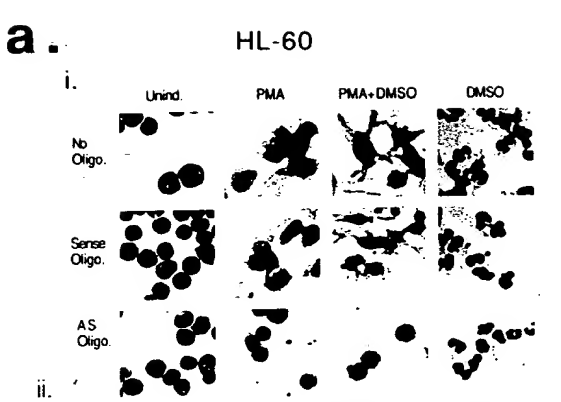
Since Egr-1 is a zinc finger transcription factor dependent on zinc metal to bind to DNA, we could assess the dependence on zinc for formation of the specific protein–DNA complex. The specific protein–DNA complex induced in HL-60 by PMA disappeared when zinc was chelated away with 1,10-phenanthroline (Phen., Figure 3a). However, this complex reappeared, almost to control levels, when different amounts of zinc were added after pretreatment with the chelator. In addition, whereas the addition of excess cold oligonucleotides containing the *Egr-1*-binding site prevented the formation of radioactive compiexes, excess cold nonspecific sequences had no effect (Figure 3a). The same types of zinc dependence and specific binding analysis were observed using nuclear extracts from HL-60 cells stimulated with PMA plus DMSO or DMSO followed by PMA, and from PMA-stimulated U-937 cells (data not shown).

Similar protein–DNA binding analysis was performed on M1 cells. A specific protein–DNA complex (Figure 3a, arrow) present at barely detectable levels using uninduced M1 cell extracts became more pronounced during macrophage differentiation induced by physiological factors (Figure 3b), consistent with the increase in steady-state levels of *Egr-1* transcripts. It can also be seen that formation of this protein–DNA complex in M1 LCM extracts was dependent on zinc (Figure 3b, lanes 5–8) and was specific for the Egr-1-binding sequence (Figure 3b, lanes 5, 9, and 10). Similar results were obtained with nuclear extracts from M1 cells induced with either IL-6 or LIF (data not shown).

Egr-1 Is Essential for Macrophage Development

Our initial interest was not only to identify but also to analyze the function of genes involved in differentiation. One way to ascertain the role of a gene is to knock out its activity and then to observe any effect on the differentiation process. Antisense *Egr-1* oligodeoxynucleotides (oligomers) in the culture medium were used to inhibit *Egr-1* expression.

As shown in Figure 4a, the presence of either the anti-



Treatment	Inducer	Cell Number ($\times 10^5$ /ml)	Differentiation Characteristics: Cell Type(%) Mature	Intermediate	Blast	Positive(%) NSE	NBT
No Oligo.	None	25.0	0.00	<1.0	>99.0	2.00	12.0
	PMA	3.50	70.0	30.0	0.00	90.0	0.00
	PMA+DMSO	3.30	82.0	18.0	0.00	89.0	0.00
	DMSO	16.0	95.0	5.00	0.00	0.00	92.0
Sense Oligo.	None	24.0	0.00	<1.0	>99.0	3.00	10.0
	PMA	3.60	71.0	29.0	0.00	91.0	0.00
	PMA+DMSO	3.40	85.0	15.0	0.00	86.0	0.00
	DMSO	15.0	94.0	6.00	0.00	0.00	89.0
AS Oligo.	None	26.0	0.00	<1.0	>99.0	2.00	11.0
	PMA	3.60	12.0	25.0	63.0	37.0	0.00
	PMA+DMSO	3.20	9.0	26.0	65.0	41.0	0.00
	DMSO	14.0	96.0	4.00	0.00	0.00	90.0

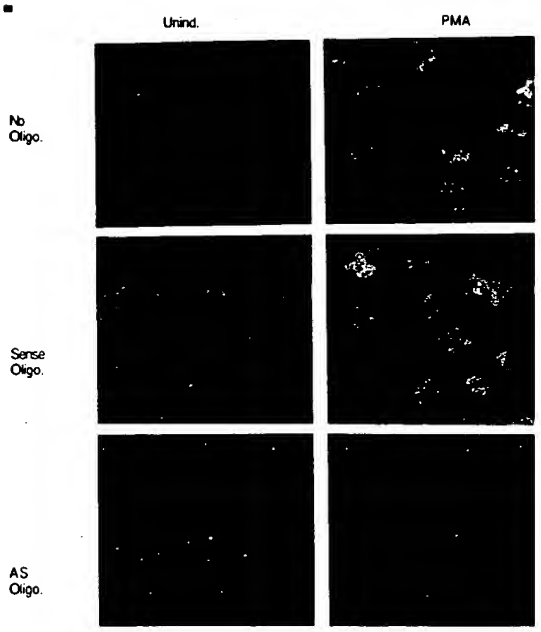


Figure 4. Effect of *Egr-1* Antisense Oligodeoxynucleotides in the Culture Medium on Differentiation of HL-60 Cells and Induction of Egr-1 Protein

(a) Effect on HL-60 cells. (i) Representative photomicrographs of HL-60 cells following May–Grunwald–Giemsa staining (× 400). (ii) Analysis of HL-60 differentiation in the absence or presence of sense or antisense oligomers. HL-60 cells (4×10^5 cells per ml) were uninduced or induced to differentiate with 2 nM PMA, with 2 nM PMA plus 1.3% DMSO for 3 days, or with 1.3% DMSO for 5 days, in the absence or presence of 80 μM sense or antisense oligomers, as described in Experimental Procedures. Cell number and morphology were determined after 3 days for untreated cells and following treatment with PMA and PMA+DMSO, and 5 days with DMSO. Cell types were determined from May–Grunwald–Giemsa-stained samples, counting at least 300 cells. Immature blast cells are characterized by scant cytoplasm and round or oval nuclei; cells at intermediate monocyte stages of differentiation are flattened, with a larger cytoplasm to nucleus ratio, and contain irregularly shaped nuclei and few interspersed or no vacuoles; granulocyte intermediates are characterized by dented but not lobulated nuclei; mature macrophage-like cells are flattened and spread out cells are interspersed with numerous vacuoles in a greatly enlarged cytoplasm; mature granulocyte-like cells are characterized by enlarged cytoplasm and lobulated nuclei. NSE staining and NBT reduction were determined after 5 days in culture, and positive cells contain red (NSE) or blue (NBT) granules in the cytoplasm. All values represent the mean of at least three independent experiments, as indicated in Experimental Procedures

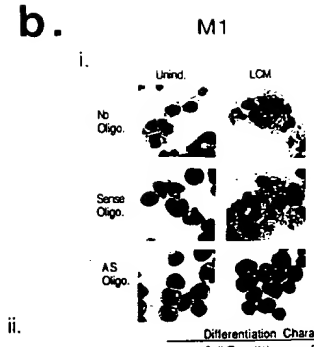
(b) Photomicrographs of HL-60 cells (× 1100) stained for Egr-1 protein by indirect immunofluorescence; note nuclear localization of the protein. Cells were treated with antisense (AS) or sense *Egr-1* oligomers (80 μM) as above (e.g., antisense/sense oligomers were added 2 hr prior to the addition of inducer). Cells were then incubated in the presence or absence of PMA (2 nM) for 3 hr and harvested for indirect immunofluorescent staining, using anti-Egr-1 antiserum as the primary antibody and fluorescein-conjugated immunoglobulin G as the secondary antibody.

sense or control sense oligomers in the culture medium did not have any effect on uninduced HL-60 cells, using either morphology (Figure 4a [i]) or cell growth (Figure 4a [ii]) as a criterion. Uninduced HL-60 cells, either in the presence or absence of oligomers, are round in morphology and nonadherent. Therefore, the oligomers had no cytotoxic effects. Analysis of uninduced HL-60 cells stained with May–Grunwald–Giemsa stain revealed their characteristically scant cytoplasm, with round nuclei. When HL-60 cells were stimulated either with PMA or PMA plus DMSO, the cells first became adherent and later flattened and spread out, acquiring a macrophage-like morphology, characterized by large cytoplasm-containing vacuoles and an irregularly shaped nucleus, as seen by May–Grunwald–Giemsa staining (Figure 4a [i]). Similar results were obtained with the control sense oligomers. However, treatment of the c lls with antisens oligomers in the presence of macrophage inducers resulted in round, adherent cells resembling undifferentiated HL-60 cells (Figure 4a [i]). As shown in Figure 4b, treatment of HL-60 cells with *Egr-1* antisense, but not sense, oligomers at the same concentration used to block macrophage differentiation also blocked induction of Egr-1 protein. Consistent with *Egr-1* not being expressed during DMSO-induced granulocytic differentiation, the oligomers did not affect granulocytic differentiation, as indicated by the figures of cells stained with May–Grunwald–Giemsa stain (Figure 4a [i]).

As shown in Figure 4a (ii), for each treatment there were similar cell numbers in the presence or absence of oligomers: approximately an 8-fold decrease for PMA and PMA plus DMSO and a 2-fold decrease for DMSO compared with uninduced HL-60. In cells induced for macrophage differentiation, there were approximately 70%–82% mature and 18%–30% intermediate cells, with no blast cells. Nearly identical results were obtained with the addition of

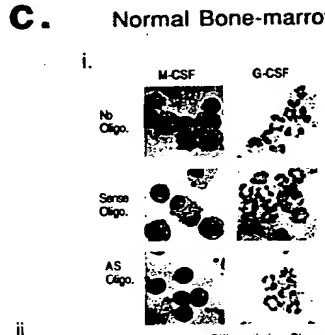
ii.

Treatment	Inducer	Cell Number ($\times 10^5$/ml)	Differentiation Characteristics: Cell Type(%) Mature	Intermediate	Blast
No Oligo.	None	16.4	0.00	5.00	95.0
	PMA	6.10	70.0	26.0	4.00
Sense Oligo.	None	15.8	0.00	6.00	94.0
	PMA	6.00	72.0	23.0	5.00
AS Oligo.	None	16.2	0.00	4.00	96.0
	PMA	5.90	10.0	25.0	65.0



ii.

Treatment	Inducer	Cell Number ($\times 10^5$/ml)	Differentiation Characteristics: Cell Type(%) Mature	Intermediate	Blast	Fc Rec. (%)	C3 Rec. (%)
No Oligo.	None	22.0	0.00	<1.00	>99.0	1.00	1.50
	LCM	2.20	74.0	23.0	3.00	55.0	74.0
Sense Oligo.	None	21.0	0.00	<1.00	>99.0	1.10	1.40
	LCM	2.00	72.0	24.0	4.00	60.0	78.0
AS Oligo.	None	20.5	0.00	<1.00	>99.0	1.20	1.60
	LCM	2.10	9.00	28.0	63.0	25.0	31.0



ii.

Treatment	Inducer	Cell Number ($\times 10^5$/ml)	Differentiation Characteristics: Cell Type(%) Macrophage Mat.	Macrophage Int.	Granulocyte Mat.	Granulocyte Int.	Blast
No Oligo.	None[a]	0.30					
	M-CSF	5.60	88.0	≤1.0	6.00	4.00	≤1.0
	G-CSF	5.40	7.00	4.00	83.0	5.00	≤1.0
Sense Oligo.	None	0.40					
	M-CSF	5.30	87.0	≤1.0	8.00	3.00	≤1.0
	G-CSF	5.80	9.00	5.00	82.0	3.00	≤1.0
AS Oligo.	None	0.40					
	M-CSF	4.30	4.00	17.0	1.00	11.0	67.0
	G-CSF	4.70	2.00	10.0	82.0	5.00	≤1.0

Figure 5. Effect of *Egr-1* Antisense Oligodeoxynucleotides in the Culture Medium on Differentiation of U-937 and M1 Myeloid Leukemia Precursor Cells and on Differentiation of Normal Myeloid Precursor–Enriched Bone Marrow Cells

(a) U-937. (i) Representative photomicrographs following May–Grunwald–Giemsa staining (× 400). (ii) Analysis of macrophage differentiation in the absence or presence of sense or antisense oligomers. U-937 cells (4 × 10^5 cells per ml) were induced for macrophage differentiation with 100 nM PMA in the absence or presence of 80 μM sense or antisense (AS) oligomers for 3 days.

(b) M1. (i) Representative photomicrographs of May–Grunwald–Giemsa-stained cytospin smears (× 400). (ii) Analysis of macrophage differentiation in the absence or presence of sense or antisense oligomers. M1 cells (2 × 10^5 cells per ml) were cultured for 3 days with 10 U/ml LCM in the absence or presence of 80 μM sense or antisense (AS) oligomers. The number of cells with Fc and C3 receptors was determined 1 day after seeding, as indicated in Experimental Procedures.

(c) Myeloid precursor–enriched bone marrow cells. (i) Representative photomicrographs of May–Grunwald–Giemsa-stained cytospin smears (× 400). (ii) Analysis of differentiation after stimulation with M-CSF or G-CSF in the absence or presence of sense or antisense (AS) oligomers. Cells (3.5 × 10^5 cells per ml) were stimulated with either M-CSF (100 U/ml) or G-CSF (160 ng/ml) in the absence or presence of 80 μM oligomers and were analyzed after 3 days in culture. In all experiments, cell types were determined as indicated in the legend to Figure 4. All values represent the mean of at least three independent experiments.

control sense oligomers to the culture medium. However, in the presence of the antisense oligomers, only 9%–12% of the cell population exhibited mature macrophage morphology, and 25%–26% exhibited intermediate morphology, with predominantly blast cells. In contrast with the effect of antisense *Egr-1* oligomers on macrophage differentiation, there was no effect on DMSO-stimulated granulocytic differentiation (Figure 4a [ii]).

In addition to morphology, we also assayed the terminal differentiation markers nonspecific esterase (NSE) (macrophage specific) and NBT reduction (granulocyte specific) (Collins, 1987; Newburger et al., 1981). Approximately 2% and 12% of the undifferentiated HL-60 cells, either with or without the oligomers, stained for NSE and exhibited NBT reduction activity, respectively. Regardless of the presence of oligomers, approximately 90% of the cells stained for NBT, but not for NSE, when stimulated with DMSO. In PMA- and PMA plus DMSO–treated HL-60 cells, approximately 90% of th cells stained positively for NSE but negatively for NBT, either in the absence or presence of the control sense oligomers. However, less than 40% of the cells treated with the antisense oligomers stained positively for NSE (Figure 4a [ii]).

Taken together, the data show that HL-60 macrophage differentiation was largely blocked in the presence of *Egr-1* antisense oligomers, whereas granulocytic differentiation was not altered. Furthermore, macrophage differentiation in the DMSO to PMA switch also was inhibited with the antisense but not with the control sense oligomers (data not shown). Although the absence of the *Egr-1* gene product inhibited macrophage differentiation, it did not prevent growth inhibition.

Similar experiments with U-937 and M1 cells have shown that, as with HL-60 cells, macrophage differentiation was specifically inhibited by *Egr-1* antisense, but not sense oligomers (Figures 5a and 5b). For M1 cells, this was further support d by the observations that antisense oligomers inhibited the appearance of Fc and C3 receptors

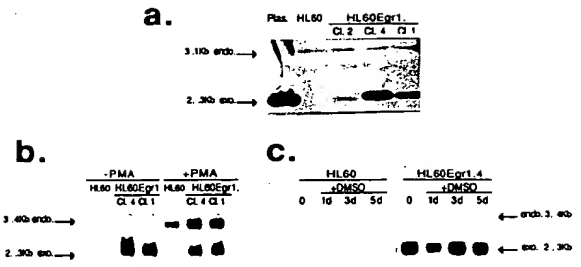


Figure 6. Establishment of HL-60 Cell Lines (HL-60Egr-1) That Constitutively Express *Egr-1* mRNA

(a) Southern blot analysis of genomic DNA demonstrating that HL-60Egr-1 cell lines (HL-60 transfected with pAC-Egr-1S) contain both the endogenous *Egr-1* gene and the exogenous *Egr-1* transgene. Twenty micrograms of genomic DNA and 0.1 ng of pAC-Egr-1S were digested with BamHI and SalI, resolved on a 1% agarose gel, transferred to gene screen plus (NEN), and hybridized to a murine *Egr-1* DNA probe. Note that HL-60 cells have only one *Egr-1* allele (Nagarajan et al., 1990).

(b) Analysis of *Egr-1* RNA expression in HL-60 and HL-60Egr-1 cell lines before and following 3 hr treatment with PMA (2 nm).

(c) Analysis of *Egr-1* RNA expression in the HL-60 and HL-60Egr-1.1 cell lines following treatment with DMSO (1.3%) for different times. RNA blots were prepared with 10 μg of RNA per lane and hybridized to a murine *Egr-1* DNA probe.

(Figure 5a [ii]), which are differentiation markers for M1 cells (Lord et al., 1991). Macrophage differentiation was observed to be repressed also in M1 cells stably transfected with a plasmid (pAC-Egr-1AS) directing constitutive high level expression of murine *Egr-1* antisense RNA (data not shown). Finally, *Egr-1* antisense, but not sense, oligomers were also observed to inhibit macrophage differentiation in primary cultures of myeloblast-enriched bone marrow cells induced with M-CSF; however, granulocytic differentiation induced with G-CSF appeared not to be inhibited (Figure 5c). As shown for HL-60 cells, treatment of U-937, M1, and normal bone marrow cells with antisense, but not sense, oligomers at the same concentration used to block macrophage differentiation also blocked synthesis of Egr-1 protein (data not shown).

Taken together, these observations suggest that removal of the *Egr-1* gene product results in inhibition of differentiation along the macrophage lineage.

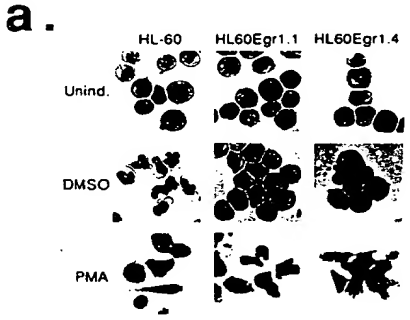
Egr-1 Is a Lineage Restriction Gene for Macrophage Development

Analysis of *Egr-1* expression in differentiation-inducible leukemic myeloblasts has shown that the *Egr-1* gene is transcriptionally silent in HL-60 cells, but active in U-937 and M1 cells (Figures 1 and 2), the latter two being predetermined for macrophage differentiation. Thus, it is important to determine if *Egr-1* plays a role in restricting differentiation along the macrophage lineage.

To test this possibility, HL-60 cells were transfected via electroporation with the vector pAC-Egr-1S, where the coding region of *Egr-1* is under control of the human β-actin promoter, to obtain HL-60 c ll lines that constitutively express an *Egr-1* transg ne (HL-60Egr-1 cell lines; Figure 6).

Southern blot analysis of genomic DNA (Figure 6a) showed two *Egr-1* hybridization bands for the HL-60Egr-1 transfectants, corresponding to the endogenous and exogenous *Egr-1* genes. Northern blot analysis was used to examine the level of expression of the xogenous murine *Egr-1* gene in two representative cell lines. As seen in Figure 6b, the unstimulated HL-60Egr-1 cell lines expressed the exogenous *Egr-1* transcript, absent in uninduced HL-60. When stimulated with PMA, the HL-60Egr-1 transfectants expressed both the endogenous and exogenous *Egr-1* transcripts, whereas HL-60 cells expressed only the endogenous *Egr-1* transcript. The level of exogenous murine *Egr-1* expression was comparable with the level of endogenous expression after 3 hr with PMA. DMSO treatment had no effect on expression of the exogenous *Egr-1* transgene (Figure 6c).

These HL-60Egr-1 cell lines allowed us to ascertain the effects of a priori expression of *Egr-1* on the ability of HL-60 cells to differentiate along the macrophage or granulocyte lineages. As shown in Figure 7, unlike HL-60 cells, HL-60Egr-1.1 and HL-60Egr-1.4 cell lines have lost the ability to be induced for granulocytic differentiation by DMSO, yet they have retained the ability to be induced for macrophage differentiation by PMA. This is evident both from cell morphology (Figures 7a and 7b) and the percentage of



b.

Clones	Inducer	Cell Number ($\times 10^6$/ml)	Differentiation Characteristics: Cell Type(%) Mature	Intermediate	Blast	Positive(%) NBT	NSE
HL-60	None	4.05	0.0	1.0	99.0	2.0	2.0
	DMSO	1.50	93.0	7.0	0.0	75.0	0.0
	PMA	0.36	73.0	27.0	0.0	0.0	90.0
HL60Egr1.1	None	3.50	0.0	1.0	99.0	2.0	2.0
	DMSO	3.45	0.0	8.0	92.0	5.0	0.0
	PMA	0.32	75.0	25.0	0.0	0.0	91.0
HL60Egr1.4	None	3.31	0.0	1.0	99.0	2.0	2.0
	DMSO	3.30	0.0	6.0	94.0	7.0	0.0
	PMA	0.33	76.0	24.0	0.0	0.0	94.0

Figure 7. Analysis of the Differentiation Characteristics of HL-60Egr-1 Cell Lines

(a) Representative photomicrographs of HL-60 and HL-60Egr-1 cell lines following May–Grunwald–Giemsa staining (× 400), before and 3 or 5 days after treatment with PMA (2 nM) or DMSO (1.3%).

(b) Analysis of HL-60 and HL-60Egr-1 cell line differentiation. Cell seeding, induction of differentiation, analysis of cell morphology, and NSE and NBT reduction was as described in the legend to Figure 4, except that cell number for proliferating untreated cells (None) and HL-60Egr-1 cell lines treated with DMSO was determined 5 days after seeding, taking into account that the cells were diluted (1:2) 3 days following seeding.

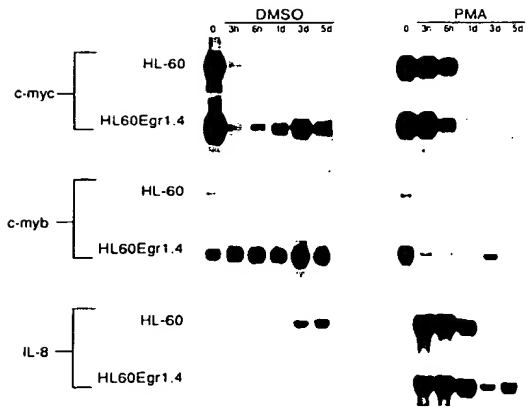


Figure 8. Analysis of c-*myc*, c-*myb*, and IL-8 Expression in HL-60 and HL-60Egr-1 Cells Following Treatment with DMSO or PMA

Expression was analyzed by hybridization of specific ^{32}P-labeled probes to Northern blots, using total RNA (10 μg per lane) extracted from cells at indicated times. Concentrations of DMSO and PMA were as indicated in the legend to Figure 7. Autoradiograms gave similar hybridization signals, comparable with that shown in Figure 1a (i), following hybridization to an actin probe.

cells scoring positive for NBT reduction and NSE staining (Figure 7b). Similar results were obtained with HL-60Egr-1.2 cells. As controls, five HL-60 cell lines obtained following transfection with a vector harboring the selectable marker only (HL-60neo), retained the ability to be induced for both granulocyte and macrophage differentiation, like parental HL-60 cells (data not shown). Antisense oligomers in the culture medium, targeted for the 5′ region of the murine cDNA transgene and spanning the AUG, partially abrogated the block in granulocytic differentiation, resulting in 15% and 24% blasts and 40% and 28% mature cells for HL-60Egr-1.1 and HL-60Egr-1.4, respectively, following treatment with DMSO. These oligomers had no effect on PMA-induced differentiation of either HL-60 or HL-60Egr-1 transfectants (data not shown).

In addition to analyzing morphological differentiation and terminal differentiation markers, we also have analyzed the expression of the proto-oncogenes c-*myc* and c-*myb*, and interleukin-8 (IL-8), which represent a set of genes that are either suppressed (c-*myc* and c-*myb*) or induced (IL-8) following induction of myeloid differentiation (Liebermann and Hoffman-Liebermann, 1989; Hoffman-Liebermann and Liebermann, 1991; Selvakumaran et al., 1992; Kowalski and Denhardt, 1989; H. O. N., unpublished data). As shown in Figure 8, expression of c-*myc* was high in uninduced HL-60 and HL-60Egr-1.4 cells. In HL-60 it decreased dramatically following 3 hr stimulation with DMSO and continued to decline to barely detectable levels by day 5, when terminal differentiation occurs. The early decline in c-*myc* expression also occurred in HL-60Egr-1.4 cells, but thereafter the expression of c-*myc* continued to increase to about half of the uninduced level by day 3 following DMSO treatment. Following PMA treatment, c-*myc* xpression d clin d to undetectable levels by day 1, in both HL-60 and HL-60Egr-1.4 cells (Figure 8). c-*myb* expression was observed to be low in uninduced HL-60 cells, decreased to undetectable levels following stimulation with DMSO, and returned to almost uninduced levels by day 5. In sharp contrast, c-*myb* expression in HL-60Egr-1.4 cells was about 50-fold higher than in HL-60 cells, and it continued to be constitutively expressed at this high level for at least 5 days following treatment with DMSO. Following treatment with PMA, c-*myb* expression was suppressed both in HL-60 and HL-60Egr-1.4 cells. Finally, it was also observed that activation of IL-8 expression, which occurred 3 days following induction of HL-60 cells for granulocytic differentiation with DMSO, was largely abolished in HL-60Egr-1.4 cells (Figure 8). In contrast, the early transient induction of IL-8, observed in HL-60 cells following stimulation for macrophage differentiation with PMA, was not impaired in HI60Egr-1.4 cells, with the exception that expression of IL-8 appeared to be prolonged. Similar results were obtained analyzing c-*myc*, c-*myb*, and IL-8 xpression in HL-60Egr-1.1 and HL-60Egr-1.2 cell lines.

Taken together, these results indicate that expression of *Egr-1* in myeloid precursor cells restricts differentiation of the cells along the macrophage lineage.

Discussion

To recapitulate the essence of the data, this work demonstrates that *Egr-1* is a macrophage, but not granulocyte, differentiation primary response gene that is essential for and restricts the differentiation of myeloid precursor cells along the macrophage lineage.

In the course of our work isolating *Egr-1* cDNA from our PMA macrophage differentiation library and demonstrating its induction during macrophage, but not granulocytic, differentiation in HL-60 and U-937 cells, others have also encountered *Egr-1* in their HL-60 macrophage differentiation library (Shimizu et al., 1991) and reported early induction of *Egr-1* in HL-60 and U-937 cells (Kharbanda et al., 1991). Our data are consistent with these data, and we have gone on to show that *Egr-1* is induced in the absence of de novo protein synthesis, exhibiting very transient kinetics of expression during PMA-stimulated macrophage differentiation of HL-60 and U-937 cells. Our studies also demonstrate that *Egr-1* is induced early in M1 leukemic and normal myeloblasts upon induction of macrophage differentiation by physiological factors. In addition to early transient expression, the results of our work show that *Egr-1* is also highly expressed in mature myeloid cells obtained as the result of physiologically induced differentiation. *Egr-1* is expressed at high levels in terminally differentiated M1 cells, following stimulation for macrophage differentiation with IL-6 or LIF, as well as in mature normal myeloid cells, including granulocytes, following induction of differentiation in primary cultures of myeloblast-enriched bone marrow cells, using G-CSF or LCM, which contains IL-6 and LIF (Hoffman-Liebermann and Liebermann, 1991). It is interesting to point out that although *Egr-1* was not expressed at detectable levels in M-CSF-induced macrophages, high levels of *Egr-1* expression were observed in these cells following stimulation with IL-6 (H. Q. N. et al., unpublished data). These observations ar

consistent with the expression of *Egr-1* in murine peritoneal macrophages and resting peripheral blood monocytes, following stimulation with GM-CSF and M-CSF, as well as with its expression in postmitotic neutrophils, following stimulation with GM-CSF or TPA (Jingwen et al., 1991; Kharbanda et al., 1991; Varnum et al., 1989b). Thus, in addition to an early role in macrophage development, *Egr-1* may also play a role in mature myeloid cells, perhaps related to the maintenance and/or regulation of a certain function(s) of the differentiated cells.

Using antisense oligomers in the culture medium (in the case of M1, cells also constitutively expressing antisense *Egr-1* transcripts) we have clearly demonstrated that *Egr-1* is essential for macrophage differentiation. However, it is clear that *Egr-1* is not sufficient for macrophage differentiation. Others have also successfully used antisense oligomers with HL-60 cells to demonstrate that M-CSF and its receptor, as well as cAMP-dependent protein kinase, are necessary for macrophage differentiation induced by PMA and cAMP analogs, respectively (Wu et al.,1990; Tortora et al., 1990, 1991). Interestingly, the inhibition of morphological monocytic differentiation with *Egr-1* antisense oligomers appeared to be more prominent than the repression of some differentiation markers such as NSE staining (Figure 4a [ii]). Also interesting is the observation with another M1 clone, which, unlike the M1 cells used in this study (Lieberman and Hoffman-Liebermann, 1989), expressed c-*fos* and appeared not to induce *Egr-1* expression following IL-6 stimulation (Shabo et al., 1990). Further studies on the role of *Egr-1* in macrophage differentiation, including identifying other genes it may interact with and/or downstream genes it regulates, will certainly be instrumental to understand these phenomena better.

We have observed that the *Egr-1* gene is transcriptionally silent in HL-60 cells, which can differentiate along either the monocytic or granulocytic lineages, but is active in U-937 and M1 cells, which are predetermined for macrophage differentiation. These observations are consistent with previous observations regarding *Egr-1* expression in U-937 cells stimulated with GM-CSF (Bernstein et al., 1991). We have shown that enforced expression of *Egr-1* in HL-60 (HL-60Egr-1) cells blocked the ability of the cells to be induced for granulocytic differentiation with DMSO or retinoic acid (unpublished data), thereby restricting differentiation of the cells to the macrophage lineage. In this regard, it is interesting that another zinc finger gene, termed *Evi-1*, has recently been shown to block granulocytic differentiation of myeloid cells (Morishita et al., 1992). Whether *Egr-1* plays a role in determining the differentiation of myeloid precursor cells along the macrophage lineage remains to be seen.

A striking difference between HL-60Egr-1 and HL-60 cells was the 50-fold higher expression of c-*myb* in HL-60Egr-1 compared with HL-60 cells. This high expression of c-*myb* was maintained in the presence of DMSO. It is interesting to note that in HL-60 variant cells, where differentiation with DMSO was observed to be reversible, c-*myb* expression was observed to be down-regulated to a barely detectable level, compared with the undetectable level in their HL-60 parental cells (Boise et al., 1992). Thus, it is possible that following stimulation of myeloid cells for differentiation, the extent of c-*myb* suppression determines whether the developmental program of differentiation is blocked, is reversible, or may proceed toward a terminal stage. It is also interesting to note that at least one consensus Egr-1-binding site is present in the c-*myb* promoter (Nicolaides et al., 1991). In addition, c-*myb* was shown to be capable of transactivating the c-*myc* promoter (Evans et al., 1990). Thus, it is also possible that the underlying mechanism by which constitutive expression of *Egr-1* blocks granulocytic differentiation involves *Egr-1*-mediated transactivation of c-*myb* expression, which in turn may transactivate c-*myc* expression.

In summary, the results presented in this work indicate that Egr-1, a zinc finger transcription factor, plays important roles in macrophage differentiation. The fact that *Egr-1* is also expressed in mature myeloid cells suggests that *Egr-1* plays additional roles in terminally differentiated cells of the myeloid lineage. These findings are consistent with observations that *Egr-1* has been localized to a region on human chromosome 5 often deleted in patients suffering from therapy-induced acute myeloid leukemia (Sukhatme et al., 1988; Nagarajan et al., 1990). Further analysis of different leukemia patients for the presence or absence of functional *Egr-1* gene products should better clarify its possible involvement in leukemic syndromes. Also, since *Egr-1* is expressed not only in cells of the myeloid lineage, but also in other cell types such as fibroblasts (Sukhatme et al., 1987; Lau and Nathans, 1987; Christy et al., 1988; Lemaire et al., 1988; Varnum et al., 1989b), B cells (Seyfert et al., 1990), and cells of neuronal origin (Milbrandt, 1987), it may also play a role in the development of other cell types. Further studies in the context of this cell system, aimed toward identifying *Egr-1* target genes, should enhance the understanding of *Egr-1* as a pleiotropic regulator of cell development and of its possible involvement in leukemic syndromes.

Experimental Procedures

Cells and Cell Cultures

Myeloblast-enriched bone marrow cells were obtained from femurs of CD-1 mice (Charles River Laboratories) injected intraperitoneally 3 days earlier with 3 ml of 10% sodium caseinate (Difco) in phosphate-buffered saline (PBS). These cells consisted of approximately 2% mature myeloid cells (segmented neutrophils, monocytes ≤1%), 61% myeloid cells at intermediate stages of differentiation (myelocytes, metamylocytes, band forms), and 33% cells at the myeloid precursor stage (myeloblasts, promyelocytes) with ≤4% of the cells of other hematopoietic lineages (lymphoid ~3%, erythroid ~1%) (Liebermann and Hoffmann-Liebermann, 1989). The M1 clone used in this study was M1D$^+$ clone 6 (Liebermann and Hoffmann-Liebermann, 1989). The HL-60 and U-937 cell lines were obtained from the American Type Culture Collection (ATCC) and recloned in our laboratory. HL-60 clone 21 and U-937 clone 14 were used throughout this study. Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO H-21, Grand Island, NY) supplemented with 10% horse serum (M1), fetal calf serum (primary bone marrow cells; cultured for 6 hr in complete medium prior to addition of myelopoietic inducers), or RPMI 1640 supplemented with 10% fetal calf serum (U-937 and HL-60 cells) plus 1% penicillin and streptomycin (GIBCO) at 37°C in a humidified atmosphere with 10% CO_2. Cells were seeded at densities as indicated, excepted for RNA extractions, where the cell concentration was adjusted to give a final density of >0.25 × 10^6 cells per ml at the time of extraction. The

number of viable cells was determined by trypan blue dye exclusion and counting in a hemocytometer.

Cytokines and Other Compounds Used

Purified human recombinant IL-6 (0.5 mg/ml) was a gift from L. Souza (Amgen, Inc., Thousand Oaks, CA) and was used at a concentration of 100 ng/ml. Purified murine LIF (10^6 U/ml) was obtained from AMRAD Corp. (Victoria, Australia) and was used at a concentration of 200 U/ml. Purified recombinant G-CSF was a gift from Amgen and was used at a concentration of 160 ng/ml. M-CSF from serum-free conditioned medium of L929 fibroblasts, concentrated 100-fold using high molecular weight polyethyleneglycol (Serva), or purified M-CSF was used at 100 U/ml. Serum-free LCM was prepared with LiCl (Horak et al., 1982) and used at a final concentration of 10%, which is equivalent to 10 U/ml differentiation-inducing activity (Liebermann and Hoffman-Liebermann, 1989). Cytokines were titrated for growth- and differentiation-inducing activities as described previously (Liebermann and Hoffman-Liebermann, 1989; Lord et al., 1991). For HL-60 cells, 1.3% DMSO and 2–10 nM PMA was used, 100 nM PMA (Sigma) was used for U-937 diluted from stock PMA solution of 100 ng/μl in DMSO (Baker) (Liebermann et al., 1981; Larsson et al., 1988).

Assays for Differentiation-Associated Properties

Morphological differentiation was determined by counting at least 300 cells on May–Grunwald–Giemsa-stained cytospin smears and scoring the proportion of immature blast cells, cells at intermediate stages of differentiation, and mature myeloid cells (Hoffman-Liebermann and Liebermann, 1991; Liebermann et al., 1981; and as indicated in figure legends). Fc and C3 receptor assays were determined as described (Lord et al., 1990a). NSE assay was determined by staining cells on plates (Nelson et al., 1983). Reduction of NBT stain was determined with a 1:1 mixture of 0.1% NBT (Sigma) dissolved in PBS and 0.2 ml of 100 ng/μl PMA in 10 ml of RPMI 1640 plus 10% fetal calf serum by incubating an equal volume of NBT solution with cells at 37°C for 25 min (modified form Torella et al., 1982). Then the mixture was cytospun on glass slides. Results of all experiments represent the mean of at least three independent determinations with standard deviations up to 15% (e.g., 11% = 11% ± 1.6%).

Construction of cDNA Library, *Egr-1* Antisense and Sense Vectors, and Other Recombinant DNA Methodologies

HL-60 cells were seeded at 0.4 × 10^6/ml and induced for macrophage differentiation with 10 nM PMA and differentiation assessed by morphology as well as NSE stain. Total RNA was extracted from cells stimulated with 10 nM PMA in the presence of 10 μg/ml cycloheximide for 3 hr using the guanidium thiocyanate method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was isolated by two cycles of fractionation over an oligo(dT)–cellulose column. cDNA was synthesized by using oligo(dT) priming for the first-strand synthesis and RNAase H plus DNA polymerase for the second strand (Gubler and Hoffman, 1983). The library was constructed using the λZAPII vector and packaged with Gigapak Gold extracts (Stratagene). The 2.3 kb EcoRI restriction fragment containing the coding region of murine *Egr-1* (Lemaire et al., 1988) was cloned into the HindIII site of pHb Apr-1-neo (Gunning et al., 1987), so that *Egr-1* was under control of the β-actin promoter. The EcoRI sites were blunt ended with T4 polymerase (IBI), and following transformation into DHf5α, tranformants were analyzed for pHb Apr-1-neo plasmid with *Egr-1* in the sense (pAC-Egr-1S) or antisense (pAC-Egr-1AS) orientation. Differential screening and cross-hybridization was accomplished using XL-1 blue cells as hosts (Lord et al., 1990b). pBluescript clones were excised using helper phage R408 according to the manufacturer (Stratagene). Minipreparations of plasmid DNA were made by the Triton lysis/lysozyme/boiling method, and large-scale plasmids were made by the cesium chloride gradient method (Hoffman-Liebermann et al., 1986; Holmes and Quigley, 1981). DNA fragments were prepared from restriction enzyme digests and isolated by agarose gel electrophoresis. Probes for human and murine *Egr-1* genes were the *Egr-1* cDNA inserts of the pBluescript plasmids HLM-38 (isolated in our lab) and MyD19 or M57/RP200 (kind gift from Dr. R. Bravo), respectively. Probe for human IL-8 was the cDNA insert of the pBluescript plasmid HLM-43 (isolated in our lab). Probes for c-*myc*, human c-*myb*, and human β-actin were the same as previously described (Liebermann and Hoffman-Liebermann, 1989; Selvakumaran et al., 1992). DNA for probes was labeled by random priming to a specific activity equal to or greater than 10^9 cpm/μg (Liebermann and Hoffman-Liebermann, 1989). Sequence analysis was performed with Bluescript SK and KS primers using the dideoxy chain termination method with modified T7 DNA polymerase (US Biochemical Corporation) and homology searches were done using GenBank database (Lord et al., 1990b).

DNA Transfections

Transfections were performed by electroporation (Bio-Rad Gene Pulsor), as described (Hoffman-Liebermann and Liebermann, 1991). In brief, a pulse was delivered to a 0.7 ml suspension containing 1.5 × 10^7 cells and 50 μg of linearized plasmid DNA. The cells were appropriately diluted and after 48 hr were seeded at 5 × 10^4/ml in growth media containing 400 μg/ml geneticin (G418 sulfate, GIBCO), and aliquoted into 24-well trays. After 3 to 4 weeks, cultures from wells containing surviving cells were expanded and then subcloned in agar in the presence of geneticin. Transfectants were maintained in a 200 μg/ml concentration of drug.

RNA Extractions, Northern (RNA) Blots, and Hybridization

RNAs were prepared as described above and 10 μg per lane was electrophoresed on 1% formaldehyde–agarose gel. Gels were denatured, neutralized, and blotted on Stratagene nylon membrane, fixed by ultraviolet irradiation, and baked for 2 hr according to the manufacturer (Stratagene). DNA for probes was labeled as described above and hybridized in 50% deionized formamide, 10% dextran sulfate, 1 M NaCl, 1% SDS, and 100 μg/ml salmon sperm DNA at 42°C with 10^6 cpm per ml of probe for 12–16 hr (Lord et al., 1990b). Blots were washed according to the manufacturer with a 60°C wash done in 0.1 × SSC and 1% SDS twice for 30 min each and exposed to prefogged X-ray film at −80°C for 48–72 hr. For quantitation, films were exposed for periods during which band intensity was linear with respect to time, and relative intensities of hybridization signals were measured at 560 nm with the gel scan program of a DU7 Beckman spectrophotometer.

Nuclear Run-On Transcription Assays

Cells (5 × 10^7) were washed three times with ice-cold PBS and then lysed on ice with lysis buffer (0.5% Nonidet P-40, 10 mM Tris [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$). After centrifugation (4°C, 500 × g) twice, nuclei were resuspended in 100 μl of glycerol storage buffer (40% glycerol, 50 mM Tris [ph 8.3], 5 mM $MgCl_2$, 0.1 mM EDTA). Run-on transcription was done at 30°C for 30 min in a 200 μl volume with reaction buffer (35% glycerol, 10 mM Tris [pH 7.5], 5 mM $MgCl_2$, 0.1 mM EDTA, 80 mM KCl, 1 mM dithiothreitol [DTT], 1 mM nucleoside triphosphates, 150 μCi of [^{32}PUTP]). Termination of transcription was accomplished by adding 10 mM $CaCl_2$, 1.5 U of of RNAase I–DNAase, and 0.4 U of RNasin (Boehringer Mannheim Biochemicals [BMB]) and incubated at 30°C for 10 min. Then, 25 μl of TES (100 mM Tris [pH 7.5], 50 mM EDTA, 5% SDS), 5 μl of 10 mg/ml tRNA, and 2 μl of 10 mg/ml proteinase K were added to the mixture and incubated for 45 min at 37°C. RNA was extracted as previously described (Chomczynski and Sacchi, 1987) and unincorporated nucleotides were removed by passing over Sephadex G-50 (fine) equilibrated in 10 mM Tris (pH 8.0), 1 mM EDTA, 100 mM NaCl. GeneScreen plus membrane containing 10 μg of linearized plasmid with or without inserts specific for actin, histone H3.3, and murine *Egr-1* or human *Egr-1* were hybridized with 2 × 10^7 cpm of nuclear run-on products, 2 ml of 1 M NaCl, 1% SDS, 50 mM Tris (pH 7.5) at 65°C for 42 hr. Hybridized strips were washed twice in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) at room temperature for 5 min each, twice in 2× SSC, 1% SDS at 65°C for 30 min each, once in 2× SSC, 10 μg/ml RNAase A at 37°C for 30 min, and twice in 0.1 × SSC, 0.1% SDS at 56°C for 30 min each (Lord et al., 1991). Strips were exposed to prefogged X-ray film at −80°C, and densitometric quantitation of signals was performed as indicated above.

Electrophoretic Mobility Shift Assays

Complementary oligodeoxynucleotides containing the Egr-1-binding sequence (5′-ATCCCGGCGCGGGGGCGAGGGCGT-3′ and 5′-ACGCCCTCGCCCCCGCGCCGGGAT-3′) were synthesized (Chemistry Department, University of Pennsylvania). Probes were made by kinasing the 0.5 μg of oligodeoxynucleotides in 50 mM Tris (pH 7.5), 10 mM

$MgCl_2$, 5 mM DTT, 50 μg/ml bovine serum albumin (BSA) with 125 μCi of [γ-^{32}P]ATP using T4 polynucleotide kinase (BMB). Probes were isolated using Sephadex G-50 (fine) as above and annealed with 10-fold excess complementary strand by heating to 65°C for 10 min, then slowly cooled to room temperature (Lemaire et al., 1990). Nuclei were prepared by incubating 5 × 10^7 cells in 400 μl of hypotonic solution (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) and lysing with 25 μl of 10% Nonidet P-40. Nuclear extracts were prepared by incubating in extraction buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) with constant agitation at 4°C for 30 min and stored at −80°C (Shreiber et al., 1989). Binding reaction mixes for gel retardation assays were set up in a 20 μl volume by incubating probes (250,000 cpm) and 3 μg of poly(dI–dC)–poly(dI–dC) with 5 μl of nuclear extracts in binding buffer (20 mM HEPES [pH 7.5], 70 mM KCl, 5 mM $MgCl_2$, 0.05% Nonidet P-40, 12% glycerol, 1 mg/ml BSA, 0.5 mM DTT, 100 μM $ZnCl_2$ [otherwise indicated]) at room temperature for 30 min. The complexes were electrophoresed on a 4% polyacrylamide gel (37:1 polyacrylamide: bisacrylamide) in 0.5× TBE (1× TBE is 89 mM Tris–borate, 89 mM boric acid, 2 mM EDTA) buffer with 150 V at room temperature. For the chelation experiments, nuclear extracts were preincubated with 1 mM 1,10-phenanthroline (Fisher) at room temperature for 10 min. Five minutes before binding reaction, specified amounts of $ZnCl_2$ were added to the pretreated extracts at room temperature, and then the binding reaction proceeded as above. Competition experiments were accomplished with a 50-fold excess of unlabeled competitor oligodeoxynucleotides, which were added concurrently with labeled probe (Cao et al., 1990).

Antisense Oligodeoxynucleotides in the Medium

Phosphorothioate-capped oligodeoxynucleotides (20 bases in length) complementary to several target sequences of 5′ untranslated region of the human *Egr-1* mRNA (Suggs et al., 1990) were compared in their efficacies to block PMA-induced morphological differentiation of HL-60 cells with a phosphorothioate-capped oligomer directed against the first five codons plus two additional bases; a combination of the HAIRPIN and the RNAFOLD programs with free energies at 37°C (PCGENE Version 6.5) was used to find 5′ sequences not engaged in snapback structures and/or potentially exposed in the secondary or tertiary structure of the mRNA (Bacon and Wickstrom, 1991). For all antisense oligomers, similar effects were obtained; however, different concentrations were required to achieve this. Sense oligonucleotides were used as controls. The phosphorothioate-capped antisense oligonucleotide (5′-TsGsCGGGGCGCGGGGAACAsCsT-3′), targeted against a 5′ sequence located 119–100 bases upstream of the AUG initiation codon, was found to be most efficient and was thus used in subsequent experiments. The phosphorothioate-capped antisense oligonucleotide (5′-GsCsGGGGTGCAGGGGCACAsCsT-3′), targeted against the homologous sequence of the 5′ untranslated region of murine *Egr-1* mRNA (120–101 bases upstream) (Sukhatme et al., 1988), was found to be equally efficient in blocking M1-induced differentiation when compared with antisense oligomers targeted against the translation initiation region, and was thus used in subsequent experiments. Specifically to block synthesis of the murine transgene protein in HL-60Egr-1 cells, the antisense oligonucleotide (^{5}TsGsCCATCCCGGACCAGCGsAsG^3) was used. This sequence was chosen owing to its limited mismatches to the homologous human sequence (4 mismatches) and to avoid going further upstream, because of the 5′ sequences, of the expression vector. Phosphorothioate-capped oligodeoxynucleotides were synthesized and obtained from Reginal DNA Synthesis Lab. (Calgary, Canada). Control sense oligodeoxynucleotides are the complementary sense strand of the human and murine antisense oligomers similarly modified. Lyophilized oligomers were resuspended in PBS without Ca^{2+}, Mg^{2+} at a 2.5 mM concentration. Cells were washed twice in serum-free medium plus 1% penicillin, 1% streptomycin (except primary bone marrow cells), and 0.45 ml aliquots were added to a 24-well plastic dish. Oligomers were added to cells with final concentration of 80 μM and incubated for 2 hr at 37°C in a humidified atmosphere with 10% CO_2. Then, serum (10% of final volume) and inducers were added. Following 24 hr the medium was replaced with new medium containing the same concentration of oligomers without or with inducer. Differentiation was assessed by observations in culture each day, by morphology, and by other differentiation markers as described above. Antisense oligomer concentrations used were the optima for inhibition of differentiation. All batches of oligomers were tested at the same concentration with uninduced HL-60, U-937, and/or M1 cells and found to have no effect on cell growth and viability, determined by trypan blue dye exclusion and counting in a hemocytometer. Results shown in the figures are representative of at least three independent experiments, each done in duplicate, using three different batches of antisense and sense oligomers. Standard deviations were up to ± 15% of the percentage values shown (e.g., 11% = 11% ± 1.6%).

Indirect Immunofluorescent Staining of Cells

Cytospin smears of cells (Shandon cytofuge, 750 rpm for 5 min) were dried at room temperature for 15 min and fixed in 3% paraformaldehyde in PBS for 10 min. Cells were lysed in 0.1% Triton X-100 in PBS and washed (three times) in PBS + 2%BSA + 0.1% NaN_3. Cells were then incubated with a 1:500 dilution (in PBS + 2% BSA) of R5232-T rabbit anti-mouse Egr-1 antiserum (Cao et al., 1990) for 90 min at room temperature in a moist chamber. Cells were washed (three times) in PBS + 2% BSA and then incubated with a 1:500 dilution of goat anti-rabbit immunoglobulin G antibodies conjugated with fluorescein (BRL) for 30 min at room temperature in the moist chamber. Cells were then washed (five times) in PBS + 2% BSA, and mounted with fluoromount. Cells were analyzed for fluorescent staining using an ultraviolet microscope (Leitz) equipped with an H3 filter, and photomicrographs were taken with the Wild-Leitz MPS46 photoautomat.

Acknowledgments

We would like to thank Drs. Peter Nowell and Premkumar Reddy for helpful suggestions in the course of this work. We would also like to thank Dr. Vikas Sukhatme for the anti-Egr-1 antibodies and for comments on the manuscript. This work was supported by National Institutes of Health grant 1RO1CA43618-01 (D. A. L.) and American Cancer Society grant IM-544 (B. H. L.). We would also like to acknowledge Amgen's support for this work.

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Received August 21, 1992; revised November 13, 1992.

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